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A multi-dimensional chromatographic approach was developed to measure the free fractions of drug enantiomers in samples that also contained a binding protein or serum. This method, which combined ultrafast affinity extraction with a chiral stationary phase, was demonstrated using the drug warfarin and the protein human serum albumin.

Many drugs and small solutes exist in multiple forms in biological systems. Drugs are often reversibly bound to carrier agents such as serum proteins in the circulatory system, causing these drugs to exist in two forms: a free fraction and bound fraction.[1–4] Because the free drug fraction is generally thought to be the biologically-active form,[3,4] there has been ongoing interest in the creation of improved tools for measuring free drug fractions and for studying drug–protein interactions in clinical and pharmaceutical samples.[1,2,5] The methods of equilibrium dialysis and ultrafiltration are often used for this work but can have long analysis times and large sample requirements (*e.g.*, equilibrium dialysis) or can introduce errors due to nonspecific adsorption to a membrane (*e.g.*, ultrafiltration or equilibrium dialysis).[6]

Chromatographic approaches based on high-performance affinity chromatography (HPAC) and ultrafast affinity extraction have recently been proposed as an alternative means for measuring free drug or free hormone fractions.[1,2,7–9] In this type of approach, small columns containing immobilized antibodies[1,2,7,8] or other binding agents, such as immobilized transport proteins,[9] are employed to extract the free form of a target drug or solute on a time scale that minimizes release of the target from its protein-bound form in a sample. Potential advantages of this approach include its speed, small sample requirements, good precision and low detection limits, especially when used with detection based on chemiluminescence or near-infrared fluorescence.[1,7,8] However, previous systems based on this method have been designed to look at only a specific drug or solute (*e.g.*, warfarin, phenytoin and thyroxine)[1,2,7–9] rather than samples that may contain multiple or related forms of the same target (*e.g.*, a mixture of drug enantiomers).

This report describes a multi-dimensional HPAC system that used ultrafast affinity extraction and chiral chromatography to simultaneously examine the free forms of drug enantiomers in complex samples (*e.g.*, serum) and to study the binding of such drugs with proteins. *R*/*S*-Warfarin and its binding protein human serum albumin (HSA) were used as models to develop and evaluate this approach. Warfarin is an anticoagulant that is often used as a racemic mixture for the treatment of thromboembolic diseases, with the *R*- and *S*-enantiomers having noticeable differences in their pharmacokinetics and protein binding properties.[3,10,11] HSA (molar mass, 66.5 kDa) is the main binding protein for warfarin and many other drugs in serum and is known to have strong interactions with both *R*- and *S*-warfarin at a region on this protein known as Sudlow site I.[10,12,13]

Fig. 1 shows the general separation and analysis strategy that was used in this study to examine the free fractions of *R*- and *S*-warfarin (see ESI† for experimental details and system configuration). Ultrafast extraction based on an immobilized HSA microcolumn was first used to separate the free and protein-bound fractions of *R*- and *S*-warfarin in the presence of a sample that contained soluble HSA. In this process, the protein-bound drug and proteins in the sample were eluted in a non-retained peak from the microcolumn, while the free drug fraction in the sample was extracted and retained. This retained free drug fraction was later eluted from the microcolumn under isocratic conditions and delivered to a second, larger HSA column, which was utilized as a chiral stationary phase for the separation and measurement of the captured drug enantiomers.[10]

Studies were first performed with this system to find the optimum flow rate conditions for extraction of the free fraction of *R*/*S*-warfarin without creating significant interferences from the

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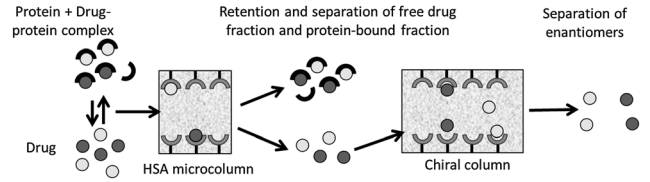

Fig. 1 General scheme for separation of the free and protein-bound fractions of a drug and resolution of the enantiomers in the free drug fraction through the use of ultrafast extraction and multi-dimensional HPAC.

portion of the drug that was originally bound to sample proteins but was released as the initial free drug fraction was removed. Fig. 2 shows the apparent free drug fractions that were obtained for warfarin–HSA mixtures at various flow rates and at 37 °C in pH 7.4, 0.067 M phosphate buffer when using a 3 mm × 2.1 mm i.d. HSA microcolumn for ultrafast affinity extraction. Based on a measured protein content of 57 (±1) mg HSA per g silica, the microcolumn was determined to contain 4.0 nmol HSA. The amount of warfarin applied per injection in Fig. 2 was 0.25% of the estimated binding capacity. Experiments with injections of only *R*/*S*-warfarin indicated that more than 99% extraction occurred for this drug's enantiomers under these injection conditions and at all of the flow rates tested in this study.

In Fig. 2, the apparent free drug fractions that were measured for a mixture of warfarin with soluble HSA were elevated at lower flow rates because the longer extraction period provided in the microcolumn under these conditions allowed for some dissociation of this drug from its protein-bound fraction in the sample, as noted previously.[1,9] This process was decreased at higher flow rates and had no significant effect on the measured free fractions at a flow rate of 5.0 mL min^{-1} or higher, which corresponded to a residence time of 100 ms or less for the sample on the HSA microcolumn.

A second set of studies examined the ability of the multi-dimensional HPAC system to separate warfarin enantiomers in the retained free drug fraction (see Fig. 3). Samples containing 1.0 μL of 5 μM warfarin or a 5 μM warfarin–10 μM HSA mixture were applied to the multi-dimensional system. The upper peak in Fig. 3(a) was obtained by injecting only warfarin onto the HSA microcolumn at 5 mL min^{-1}. The free fraction of warfarin that was extracted by this microcolumn eluted with a retention time of approximately 17–20 s. This figure also shows a

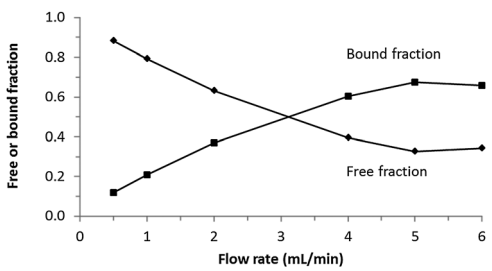

Fig. 2 Effect of flow rate on the measurement of free fractions in mixtures of racemic warfarin and HSA when using ultrafast affinity extraction. Conditions: 1.0 μL of 10 μM warfarin or 10 μM warfarin–20 μM HSA injected onto a 3 mm × 2.1 mm i.d. HSA microcolumn.

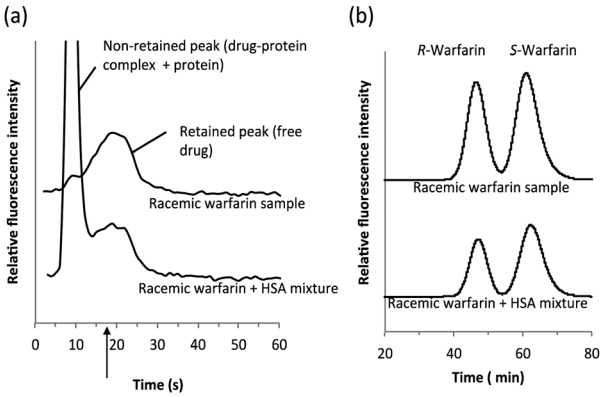

Fig. 3 Chromatograms for 1.0 μL injections of 5 μM racemic warfarin or 5 μM racemic warfarin plus 10 μM HSA on (a) an HSA microcolumn at 5.0 mL min^{-1} or (b) a chiral HSA column at 0.5 mL min^{-1} after sample passage through the HSA microcolumn. The arrow in (a) shows the time at which a valve was switched to pass the retained components from the HSA microcolumn onto the larger HSA column that was used for the chiral separation. These results were obtained at 37 °C using pH 7.4, 0.067 M phosphate buffer as the mobile phase for both columns.

chromatogram that was obtained when the same amount of warfarin and a two-fold mol excess of soluble HSA were injected onto the HSA microcolumn under identical conditions. In this case, a large non-retained peak was now observed at 5–7 s due to the elution of the soluble HSA and protein-bound fraction of warfarin. The second retained peak in this case represented the free fraction of warfarin that was extracted by, and later eluted from, the HSA microcolumn.

The free warfarin fraction, or a representative portion of this fraction, that was extracted by the HSA microcolumn was then eluted in the presence of the pH 7.4 phosphate buffer onto a 5 cm × 2.1 mm i.d. HSA column at 0.5 mL min^{-1} for a chiral separation. The chromatograms in Fig. 3(b) show the peaks that were obtained for *R*- and *S*-warfarin in samples that contained only racemic warfarin or racemic warfarin plus soluble HSA. With this combined approach, it was possible to simultaneously study the free fractions for both *R*- and *S*-warfarin in the original sample. Although the chiral separation obtained with the pH 7.4 buffer in Fig. 3(b) provided baseline resolution for the warfarin enantiomers and was sufficient for these exploratory studies, the speed of this latter step could be reduced to less than 10 min by using a more efficient HSA monolith column and/or by adding an organic modifier to the mobile phase (see ESI† for examples).[10,14,15]

The free fraction of *R*- or *S*-warfarin in a mixture of racemic warfarin and soluble HSA was calculated by dividing the concentration of the enantiomer's free fraction, as represented by lower peaks in Fig. 3(b), by the concentration measured for the enantiomer and at the same total sample concentration but with no soluble protein present, as represented by the upper peaks in Fig. 3(b). The concentrations of the *R*- and *S*-warfarin fractions were determined by comparing the sizes of these peaks to those obtained with the same chromatographic system and using warfarin standards. The free fractions for *R*- and *S*-warfarin in various samples were measured by this approach based on multi-dimensional HPAC and by employing a reference method that made use of ultrafiltration followed by a

Table 1 Free drug fractions measured for *R*- and *S*-warfarin in samples containing soluble HSA or human serum

	Measured free fraction[a]	
Sample & Analyte	Multi-dimensional HPAC	Ultrafiltration + chiral separation
Racemic warfarin (5 μM) + HSA (10 μM)		
R-Warfarin	42 (±4)%	41 (±4)%
S-Warfarin	38 (±5)%	32 (±3)%
Racemic warfarin (30 μM) + HSA (600 μM)		
R-Warfarin	1.8 (±0.8)%	1.5 (±0.5)%
S-Warfarin	1.3 (±0.2)%	1.8 (±0.2)%
Racemic warfarin (30 μM) + human serum[b]		
R-Warfarin	2.7 (±1.7)%	2.5 (±0.2)%
S-Warfarin	1.1 (±0.3)%	1.6 (±0.7)%

[a] These values were obtained at 37 °C in pH 7.4, 0.0067 M potassium phosphate buffer. The numbers in parentheses represent ±1 S.D. ($n = 3$). [b] The human serum contained approximately 600 μM HSA.

Table 2 Association equilibrium constants estimated for *R*- and *S*-warfarin with soluble HSA based on a single-site binding model

	Association equilibrium constant, K_a[a] (M^{-1})		
Sample & Analyte	Multi-dimensional HPAC	Ultrafiltration + chiral separation	Literature value [ref. 10]
Racemic warfarin (5 μM) + HSA (10 μM)			
R-Warfarin	$2.0\ (\pm0.4) \times 10^5$	$2.1\ (\pm0.4) \times 10^5$	$2.1\ (\pm0.2) \times 10^5$
S-Warfarin	$2.4\ (\pm0.6) \times 10^5$	$3.2\ (\pm0.4) \times 10^5$	$2.6\ (\pm0.4) \times 10^5$

[a] These values were obtained at 37 °C in pH 7.4, 0.0067 M potassium phosphate buffer. The numbers in parentheses represent ±1 S.D. ($n = 3$).

chiral separation using an HSA column (see ESI† for experimental details). The results that were obtained by each method are summarized in Table 1.

Table 1 shows that there was good agreement between the free fractions that were measured in Fig. 3 by the multi-dimensional HPAC method and by ultrafiltration followed with a chiral separation. For a sample that contained 5 μM racemic warfarin and 10 μM soluble HSA, the absolute difference in these free fractions was 1–6%, with the results showing no significant difference at the 95% confidence level. In addition, these measured free fractions agreed with the range of 33–43% that was predicted for *R*- and *S*-warfarin based on the known binding constants of this system.[9,10]

The multi-dimensional HPAC method was also used to examine other samples. One of these samples contained 30 μM racemic warfarin and 600 μM HSA, representing a clinically relevant concentration of HSA and a typical therapeutic concentration for warfarin.[16] Good agreement between the multi-dimensional HPAC system and reference method was again obtained, with an absolute difference in the measured free fractions of 0.3–0.5% and no significant differences at the 95% confidence level. In addition, the measured free fractions were consistent with a range of approximately 0.5–2% that was estimated from the reported binding constants for the warfarin–HSA interaction.[9,10] The free fractions of the warfarin enantiomers were also examined in human serum that was spiked with a therapeutic level of this drug. The results for the multi-dimensional HPAC system and reference method were again comparable, with an absolute difference of 0.2–0.5% and no significant differences being noted at the 95% confidence level.

The multi-dimensional HPAC system was next used as a screening tool to determine the association equilibrium constants for *R*- and *S*-warfarin with HSA. This was accomplished by using the free fraction data along with a single-site binding model (see ESI† for details). Table 2 shows the results that were obtained for the 5 μM warfarin–10 μM HSA mixture. This table also lists previous binding constants that have been reported for *R*- and *S*-warfarin at Sudlow site I of HSA under the same pH and temperature conditions.[10] Under these conditions, the association equilibrium constants that were estimated by multi-dimensional HPAC and the reference methods continued to show good agreement at the 95% confidence level with one another and with the literature values.

Similar calculations to those used in Table 2 were carried out for the 30 μM warfarin–600 μM HSA mixture and for the spiked serum samples (see ESI, Table S1†). The association equilibrium constants that were obtained by multi-dimensional HPAC and the reference approach were again consistent with each other; however, the estimated binding constants were 38–71% smaller than the literature values. This latter difference is probably due to the greater uncertainty that was present for these samples in the measurement of their relatively small free fractions. It is also possible there were some deviations from a single-site binding model due to greater nonspecific interactions by warfarin with the much larger amounts of HSA, and other proteins in the case of the serum, in this second group of samples.

In summary, it was shown that a multi-dimensional HPAC system that used ultrafast affinity extraction in combination with a chiral separation could be used to simultaneously measure the free fractions of *R*- and *S*-warfarin in serum or drug–protein mixtures. This approach was also used to estimate the binding constants for these enantiomers with HSA. The results of this method gave good agreement with a reference method that was based on ultrafiltration plus a chiral separation. However, the multi-dimensional HPAC method had several potential advantages over ultrafiltration. For instance, this method required only 1 μL of sample per injection and could isolate the free warfarin fractions within 20–30 s of injection. In comparison, a 1 mL sample was needed for ultrafiltration and 1 h was required for the separation of a free drug fraction by this approach. It was also possible to directly couple the ultrafast extraction with a chiral separation to automate and complete both steps using a single system. This approach is not limited to warfarin or HSA but could easily be extended to other chiral drugs, or drug mixtures, and their binding proteins through the use of similar affinity microcolumns and chiral stationary phases.

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Notes and references

1 W. Clarke, J. E. Schiel, A. Moser and D. S. Hage, *Anal. Chem.*, 2005, **77**, 1859–1866.

2 W. Clarke, A. R. Chowdhuri and D. S. Hage, *Anal. Chem.*, 2001, **73**, 2157–2164.

3 H. Takahashi, T. Kashima, S. Kimura, N. Muramota, H. Nakahata, S. Kubo, Y. Shimoyama, M. Kajiwara and H. Echizen, *J. Chromatogr., B: Biomed. Sci. Appl.*, 1997, **701**, 71–80.

4 M. Rowland and T. N. Tozer, *Clinical pharmacokinetics, concepts and applications*, Lippincott, Philadelphia, PA, 1995.

5 F. Herve, S. Urien, E. Albengres, J. C. Duche and J. P. Tillement, *Clin. Pharmacokinet.*, 1994, **26**, 44–58.

6 J. W. Melten, A. J. Wittebrood, J. J. Hubb, G. H. Faber, J. Wemer and D. B. Faber, *J. Pharm. Sci.*, 1985, **74**, 692–694.

7 J. E. Schiel, Z. Tong, C. Sakulthaew and D. S. Hage, *Anal. Chem.*, 2011, **83**, 9384–9390.

8 C. M. Ohnmacht, J. E. Schiel and D. S. Hage, *Anal. Chem.*, 2006, **78**, 7547–7556.

9 R. Mallik, M. J. Yoo, C. J. Briscoe and D. S. Hage, *J. Chromatogr., A*, 2010, **1217**, 2796–2803.

10 B. Loun and D. S. Hage, *Anal. Chem.*, 1994, **66**, 3814–3822.

11 *Applied pharmacokinetics: principles of therapeutic drug monitoring*, ed. W. E. Evans, J. J. Schentag and W. J. Jusko, Applied Therapeutics, Vancouver, WA, 1992.

12 X. M. He and D. C. Carter, *Nature*, 1992, **358**, 209–215.

13 G. Sudlow, D. J. Birkett and D. N. Wade, *Mol. Pharmacol.*, 1976, **12**, 1052–1061.

14 R. Mallik and D. S. Hage, *J. Pharm. Biomed. Anal.*, 2008, **46**, 820–830.

15 E. L. Pfaunmiller, M. Hartmann, C. M. Dupper, S. Soman and D. S. Hage, *J. Chromatogr., A*, 2012, **1269**, 198–207.

16 *Tietz textbook of clinical chemistry and molecular diagnostics*, ed. C. A. Burtis, E. R. Ashwood and D. E. Bruns, Saunders, St. Louis, MO, 2006.

SUPPLEMENTARY INFORMATION

Analysis of Free Fractions for Chiral Drugs using Ultrafast Extraction and Multi-Dimensional High-Performance Affinity Chromatography

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EXPERIMENTAL SECTION

Materials and Reagents. The HSA (Cohn fraction V, essentially fatty acid free), human serum (from male AB plasma, H4522, lot 039K0728; sterile filtered and tested negative for HIV-1/HIV-2, hepatitis B and hepatitis C), and racemic warfarin (98% pure) were from Sigma (St. Louis, MO, USA). The reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). The Nucleosil Si-300 silica (7 μm particle diameter, 300 Å pore size) was purchased from Macherey Nagel (Dűren, Germany). All buffers and aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and were passed through Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA)

Apparatus. The affinity columns were packed using a Prep 24 preparative pump from ChromTech (Apple Valley, MN, USA). The chromatographic system consisted of a PU-2080 Plus HPLC pump from Jasco (Easton, MD, USA), two six-port Lab Pro valves (Rheodyne, Cotati, CA, USA), and a Shimadzu RF-10AXL fluorescence detector (Kyoto, Japan). An Alltech water jacket (Deerfield, IL, USA) and an Isotemp 3013D circulating water bath from Fisher were used to maintain a temperature of 37.0 (±0.1) °C for the columns during all experiments described in this report. The chromatographic data were collected and processed using in-house programs written in LabView 5.1 (National Instruments, Austin, TX, USA). The ultrafiltration studies were performed using a 5702RH centrifuge from Fisher and tubes containing Ultracel YM-T cellulose membranes (30 kDa cut-off), as obtained from Millipore (Billerica, MA, USA).

Column preparation. The stationary phase used in these studies consisted of HSA that was immobilized on Nucleosil Si-300 silica by the Schiff base, as performed according to the literature.[1] Control supports were prepared in the same manner but with no HSA being added during the immobilization step. The protein content of the final HSA support was determined in

triplicate by a BCA assay using HSA as the standard and the control support as the blank.

An HPLC column packer was used to place the supports into stainless steel columns with dimensions of 1 cm × 2.1 mm i.d. or 5 cm × 2.1 mm i.d. The columns with dimension of 3 mm × 2.1 mm i.d. used a frit-in-column design, as described in Ref. 2. The longer columns were prepared using traditional stainless steel HPLC housings and end fittings. The packing solution for all of these columns was pH 7.4, 0.067 M potassium phosphate buffer, and the packing pressure was 4000 psi (28 MPa).

Chromatographic studies. This work utilized a multi-dimensional system in which a 3 mm × 2.1 mm i.d. HSA microcolumn was used for the extraction of free drug fractions and a 5 cm × 2.1 mm i.d. HSA column was used for chiral separations of the retained free drug fractions. The sample loading, injection and column switching were controlled by two separate Rheodyne six port valves (Cotati, CA, USA), as illustrated in Fig. S1. The mobile phase was pH 7.4, 0.067 M potassium phosphate buffer in the free drug extractions and in the initial chiral separations. All samples containing racemic warfarin and HSA were prepared in this buffer and incubated for at least 1 h before injection to allow equilibrium to be established between the free and protein-bound fractions of the drug in the sample.[3]

The initial studies examining the free fraction extraction of *R*- and *S*-warfarin used a 1.0 μL sample of 10 μM racemic warfarin or a 10 μM racemic warfarin/20 μM HSA mixture that was injected onto a 3 mm × 2.1 mm i.d. HSA microcolumn at flow rates ranging from 0.5 mL/min to 6.0 mL/min. In the final method that was developed in this study, a 1.0 μL sample injection was made onto the 3 mm × 2.1 mm i.d. HSA microcolumn at a flow rate of 5.0 mL/min for extraction of the free drug fraction. Eighteen seconds later, a switching valve was used to transfer the eluting free drug fraction to a longer 5 cm × 2.1 mm i.d. HSA column for use in a

chiral separation at 0.5 mL/min. The aqueous samples used in these latter studies contained 5 μM racemic warfarin or a 5 μM warfarin/10 μM HSA mixture; 30 μM racemic warfarin or 30 μM warfarin/600 μM HSA, to examine the use of this method at clinically-relevant concentrations; and a mixture of 30 μM racemic warfarin and human serum (which contained approximately 600 μM HSA) to study the feasibility of using this system with human serum samples. The warfarin enantiomers were detected by monitoring their fluorescence at an excitation wavelength of 310 nm and an emission wavelength of 390 nm. The concentrations of *R*- and *S*-warfarin in each sample were determined by comparing the resulting peak areas to those obtained for warfarin standards.

Ultrafiltration studies. Before sample introduction, each ultrafiltration device was washed three times with 1 mL water and spun at 1500 × g for 5 min. The devices were then washed three times in the same manner with 1 mL of pH 7.4, 0.067 M potassium phosphate buffer. Any remaining buffer in the device was removed by spinning the filtration device at 1500 × g for 15 min. Immediately after these washing and pretreatment steps, a 1 mL sample containing warfarin or warfarin plus HSA, as prepared in pH 7.4, 0.067 M potassium phosphate buffer or human serum, was introduced into three ultrafiltration devices and spun at 1500 × g and 37ºC for 2.5 min or 6.0 min, respectively (Note: different spinning periods were used to make sure that no more than 0.5 mL of the sample passed into the filtrate vial, thus allowing for accurate free drug fraction measurements).[4]

The resulting filtrates were collected for the measurement of their warfarin concentrations by using an HPLC-based chiral separation. This was accomplished by making a 5 μL injection of each filtrate sample at 1.0 mL/min onto a 1 cm × 2.1 mm i.d. HSA column. The mobile phase in this case consisted of pH 7.4, 0.067 M potassium phosphate buffer containing

1.5% (v/v) 1-propanol. The elution of warfarin enantiomers from this column was again monitored by using a fluorescence detector, as described in the previous section, and the concentrations of *R*- and *S*-warfarin in each filtrate were determined by comparing the resulting peaks areas to those that were obtained by the same approach when using warfarin standards.

Prediction of Free Fractions from Association Equilibrium Constants. For a drug and protein interaction that involves 1:1 binding, the relationship between the theoretical free fraction (F) and the association equilibrium constant (K_a) for this interaction can be described by using eqs (1) and (2),

$$F = \frac{C_d - [D-P]}{C_d} \quad (1)$$

$$K_a = \frac{[D-P]}{(C_d - [D-P])(C_p - [D-P])} \quad (2)$$

in which C_d is the total concentration of drug in the original sample, C_p is the total concentration of protein in the sample, [*D*-*P*] is the concentration of the drug-protein complex in the original sample.[3]

In this study, both *R*- and *S*-warfarin were present in a sample containing racemic warfarin and both enantiomers were able to interact with any HSA that was present. Thus, the free fractions for these two enantiomers (F_R and F_S) and their association equilibrium constants ($K_{a,R}$ and $K_{a,S}$) were calculated separately, as described by eqs (3)-(6),

$$F_R = \frac{C_R - [R-P]}{C_R} \quad (3)$$

$$K_{a,R} = \frac{[R-P]}{(C_R - [R-P])(C_p - [R-P] - [S-P])} \quad (4)$$

$$F_S = \frac{C_S - [S-P]}{C_S} \quad (5)$$

$$K_{a,S} = \frac{[S-P]}{(C_S - [S-P])(C_p - [R-P] - [S-P])} \quad (6)$$

where C_R and C_S represent the concentrations of *R*- and *S*-warfarin in the original sample. According to the information provided by their supplier, the *R*- and *S*-warfarin were present in identical amounts in their original racemic mixture. Under these conditions, the relationship of their concentrations with C_d can be described by eq (7).

$$2\ C_R = 2\ C_S = C_d \qquad (7)$$

In the initial studies examining use of the multi-dimensional HPAC method to measure free fractions, the sample consisted of 2.5 μM *R*-warfarin (C_R), 2.5 μM *S*-warfarin (C_S) and 10 μM HSA (C_P). The association equilibrium constants ($K_{a,R}$ and $K_{a,S}$) of *R*- and *S*-warfarin with HSA have been reported to be 2.1 (± 0.2) $\times 10^5$ and 2.6 (± 0.4) $\times 10^5$ M^{-1} under the same pH and temperature conditions as used in this current study.[5] Substituting these values into eqs (3)-(7), gave predicted free fractions for *R*- and *S*-warfarin of 0.41 (± 0.02) and 0.36 (± 0.03), respectively, as found by using the Solver function in Microsoft Excel. A similar process was used to estimate free fractions for the other samples that were examined in this study.

Estimation of Association Equilibrium Constants from Measured Free Fractions. Based on the free drug fractions that were measured in this study, it was possible to estimate the overall association equilibrium constants for each drug with HSA. This was accomplished by using the free fraction data and the equations introduced in the previous section. For instance, by using eq (3) the concentration of the *R*-warfarin/HSA complex ([*R*-*P*]) could be calculated from the measured free fraction of *R*-warfarin (F_R). Substituting the value of [*R*-*P*] into eq (4) then made it possible to obtain $K_{a,R}$. [3] The same process was employed for the calculation of $K_{a,S}$ by using eqs (5) and (6). The results that were obtained by this process are shown in both Table 2 in the main body of the paper and in Table 1S in this Supplementary Information.

Use of Alternative Conditions for the Chiral Separation of *R*- and *S*-Warfarin. The

results shown in Fig. 3(b) in the main body of this paper were based on the use of pH 7.4, 0.067 M phosphate buffer as a mobile phase for both of the HSA columns in the multi-dimensional HPAC system. This was done for the sake of simplicity in the initial design of this system and resulted in the type of separation that is shown in Fig. S2(a) and Fig. 3(b). However, it was also possible to conduct chiral separations by using small amounts of organic modifiers in the mobile phase to further improve this separation. For instance, Fig. S2(b) shows the effects of adding 1.5% (v/v) 1-propanol to the mobile phase while keeping all of the other conditions the same as in Fig. S2(a). This change lead to a large increase in resolution but lowering the retention factor for *R*-warfarin and increasing the retention factor for *S*-warfarin, as noted previously for similar HSA columns.[5]

Other conditions could also be used to improve this chiral separation. As an example, using the same flow rate and amount of 1-propanol as in Fig. S2(b) but decreasing the HSA column size to 1 cm × 2.1 mm i.d. still gave baseline resolution between *R*- and *S*-warfarin but decreased the separation time to around 18 min at 0.50 mL/min, as shown in Fig. S2(c). Using 1.5% 1-propanol in the mobile phase and increasing the flow rate to 1.0 mL/min with the 1 cm × 2.1 mm i.d. HSA column also gave baseline resolution, but with the separation now being complete in roughly 9 min (see Fig. S2(d)). Another way to improve this separation would be to use an alternative support, such as a monolith column. For instance, a separation of *R*- and *S*-warfarin in 6 min was recently reported when using a 1 cm × 4.6 mm i.d. HSA monolith column based on a co-polymer of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) that was operated at 2.0 mL/min and that used 0.5% 1-propanol in a pH 7.4, 0.067 M phosphate buffer as the mobile phase.[6]

References

1 C. M. Ohnmacht, J. E. Schiel, D. S. Hage, *Anal. Chem.* **2006,** *78,* 7547-7556.

2 J.E. Schiel, Ph.D. Thesis, University of Nebraska-Lincoln, Lincoln, NE, 2009.

3 R. Mallik, M. J. Yoo, C. J. Briscoe, D. S. Hage, *J. Chromatogr. A,* **2010,** *1217,* 2796-2803.

4 J.B. Whitlam, K.F. Brown, *J. Pharmaceut. Sci.* **1980**, *70,* 146-150.

5 B. Loun, D. S. Hage, *Anal. Chem.* **1994,** *66,* 3814-3822.

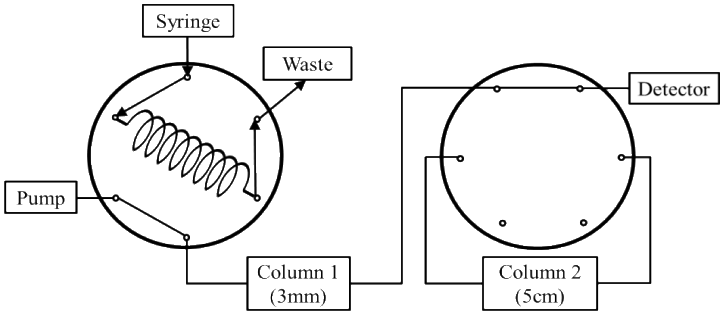
6 E. L. Pfaunmiller, M. Hartmann, C. M. Dupper, S. Soman, D. S. Hage, *J. Chromatogr. A*, 2012, **1269**, 198-207.

FIGURE LEGENDS

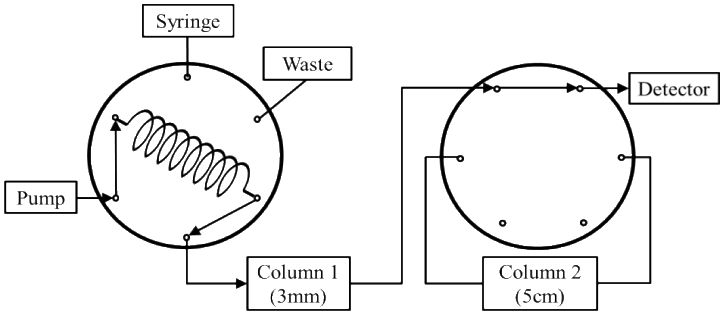
Fig. S1 Valve configurations used in the multi-dimensional HPAC system. The first valve was used for (a) loading and (b) injecting a sample onto an HSA microcolumn for a free fraction separation. (c) The second valve was switched when the protein-bound drug complex and excess protein had been passed through the HSA microcolumn and the free drug fraction had just begun to exit this microcolumn. The free drug fraction, or a representative portion, was then passed on to a second and longer HSA column for a chiral separation.

Fig. S2 Chromatograms for injections of racemic warfarin solution onto HSA columns for chiral separation under different conditions. The results shown in (a) were obtained at 0.5 mL/min by injecting 5 μL of 30 μM racemic warfarin onto 5 cm × 2.1 mm i.d. HSA column in the mobile phase of pH 7.4, 0.067 M potassium phosphate buffer. The results shown in (b) were obtained for the same sample and column with (a) in the mobile phase containing 1.5% 1-propanol as modifier. The results shown in (c) were obtained for the same sample and mobile phase with (b) on to a 1 cm × 2.1 mm i.d. HSA column. The results shown in (d) were obtained for the same sample, mobile phase and column with (c) when the flow rate is 1.0 mL/min. The HSA columns were prepared by the Schiff base method. And these chromatographic studies were finished at the temperature of 37 ℃.

(a) Sample loading



(b) Sample injection and free drug/protein-bound drug separation



(c) Chiral separation of free drug fraction

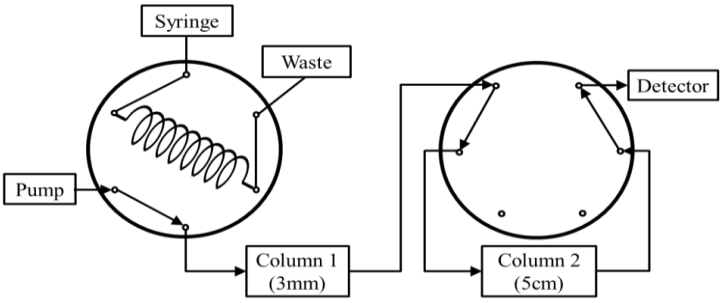


Fig. S1

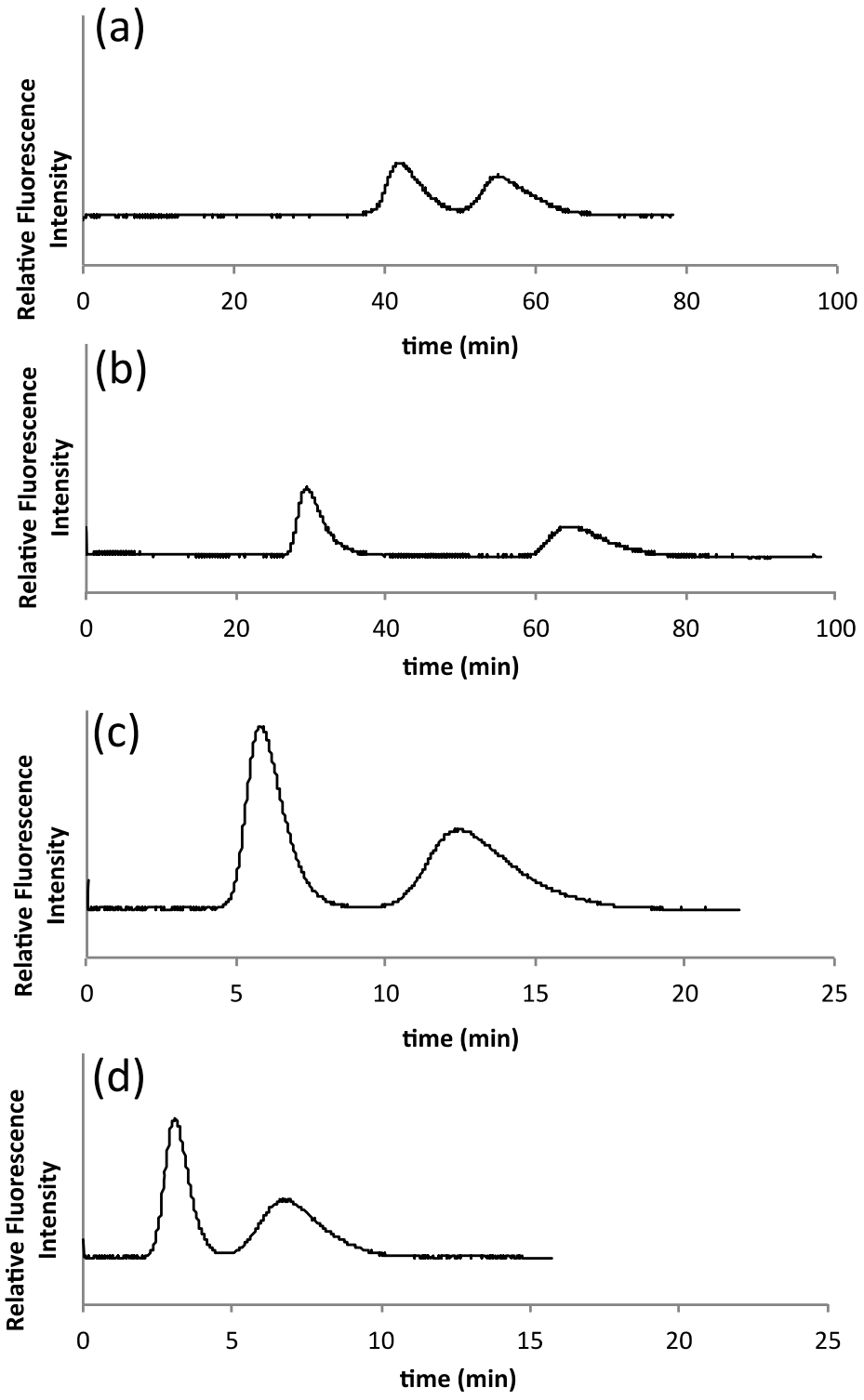


Fig. S2.

Table S1. Association equilibrium constants for *R*- and *S*-warfarin with soluble HSA

Sample & Analyte	Association equilibrium constant, K_a (M^{-1})[a]		
	Multi-dimensional HPAC	**Ultrafiltration + chiral separation**	**Literature value [Ref. 5]**
Racemic warfarin (30 μM) + HSA (600 μM)			
R-Warfarin	1.0 (± 0.4) ×10^5	1.2 (± 0.4) ×10^5	2.1 (± 0.2) ×10^5
S-Warfarin	1.3 (± 0.2) ×10^5	0.9 (± 0.1) ×10^5	2.6 (± 0.4) ×10^5
Racemic warfarin (30 μM) + Human serum[b]			
R-Warfarin	0.6 (± 0.4) ×10^5	0.7 (± 0.1) ×10^5	2.1 (± 0.2) ×10^5
S-Warfarin	1.6 (± 0.5) ×10^5	1.1 (± 0.5) ×10^5	2.6 (± 0.4) ×10^5

[a]These values were measured for the given samples at 37 °C in pH 7.4, 0.067 M potassium phosphate buffer. The numbers in parentheses represent a range of ±1 S.D. Values from Ref. 5 were measured under the same conditions by using frontal analysis.

[b]The human serum contained approximately 600 μM HSA.